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OPTIMIZATION OF CONDITIONS FOR CAROTENOID EXTRACTION FROM SHRIMP WASTE USING ORGANIC SOLVENT

Le Thanh Ha*, Le Duy Thang

School of Biotechnology and Food Technology, Hanoi University of Science and Technology, 1 Dai Co Viet, Hai Ba Trung, Ha Noi

*Email: *ha.lethanh@hust.edu.vn*

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Abstract. In this study, factors affecting the extraction yield of carotenoids from dried shrimp waste by organic solvents have been studied. The results showed that the solvent ratio hexane: acetone = 3: 1 gave the highest carotenoid yield. At this ratio of solvent's mixture, the carotenoid yield reached highest at temperature 60 °C after 2 hours extraction, which was 44.64 µg/g raw shrimp waste (d.b.) (ratio of solvent to raw material 3/1). Ultrasound or vortexing gave higher extraction yield than in static conditions, which was 1.8- fold to 1.5- fold increase, respectively. At the ratio of solvent: dried shrimp waste = 4: 1, the amount of carotenoids recovered at 60 °C for 2 hours with vortexing increased to 57.4 µg/g. However, if the shrimp waste was hydrolyzed with Alcalase at 50 °C for 4 hours before extraction by solvent, the amount of recovered carotenoids achieved 148.8 µg/g of raw material.

Keywords: Alcalse, Carotenoids, *Penaeus vannamei*, shrimp waste, extraction.

Classification numbers: 1.3.1, 1.5.1

1. INTRODUCTION

Viet Nam is the world's third largest shrimp producer. Shrimp production reached 762,000 tones in 2018, which was 6.3 % increase compared to 2017. According to forecasting of Vietnam's seafood exportation, the shrimp production will be approximately 900 thousand tons by 2020; 1.5 million tons by 2030. The processed shrimp generates an industrial waste which accounts up to 40 to 50 % (w/w) of raw material. As a result, an estimated 350,000 tons (wet weight) of shrimp waste is released per year. The shrimp waste can be used to obtain carotenoids, which are bioactive substances highly demanded by the food and pharmaceutical industries. The carotenoids attracted interest of many researchers due to their natural origin, null toxicity and high versatility.

Carotenoids could be extracted from shrimp waste by organic solvent. A 50:50 mixture of isopropyl alcohol and hexane gave the highest (43.9 µg/g waste) extraction yield of carotenoids compared to acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, hexane individually and to a 50:50 mixture of acetone and hexane [1]. Vegetable oils have also been successfully used as solvents for the extraction of carotenoid

components from vegetal sources and crustaceans. Sachindra *et al.* [2] reported that the carotenoid recovery yield in shrimp waste with different oils were from 16.1 µg/g to 26.1 µg/g waste, which was much lower than that with organic solvent. In recent years, supercritical CO_2 (SC - CO_2) has been used as an alternative method for carotenoid extraction. The extracting yield was low due to their low solubility in supercritical CO_2 [3] and an economical constraint due to the high investment cost inherent to high pressure processes [4]. Proteolytic hydrolysis has been used for recovery of carotenoids from shrimp waste and had positive effect on the carotenoid yield [5, 6].

Many studies on extraction of carotenoids from fresh shrimp waste were reported. According to Trang study, the carotenoids yield from fresh shrimp waste decrease 28.3 % after 1 month storing even during cold storage at -20 °C [7]. Mezzomo reported in his study, that the extracted yield of carotenoids from dried shrimp waste increased in comparison to fresh shrimp waste [8]. Therefore, drying could be alternative storage to cold storage. The aim of this study is to find the optimum condition for carotenoid extraction from dried shrimp waste.

2. MATERIALS AND METHODS

2.1. Materials

The raw material consists of whiteleg shrimp (*Penaeus vannamei*) waste, composed essentially by head and carpace. The protein contents, ash contents and moisture contents of raw material were 12.07 ± 0.21 %, 4.26 ± 0.22 %, and 77.87 ± 0.46 %, respectively. The residues were provided by Hai Phong seafood export company Ltd, Hai Phong, Vietnam. The fresh shrimp residues after processing were directly transported to laboratory under iced conditions and were stored at -20 °C for subsequent use.

2.2. Method

2.2.1. Pre-treatment of shrimp waste

The shrimp waste was submitted to the following pretreatments: a) drying with subsequent grinding b) cooking with subsequent drying and grinding; c) proteolytic hydrolysis.

For cooking, the raw material was submitted to a quick cooking in a heating bath with water at 100 ^{o}C for 10 min. For drying, the shrimp waste was dried at 60 ^{o}C for 5 h in an oven with air circulation. The moisture content of died shrimp waste was 4.5 %. For grinding, the dried shrimp waste was grinded in a domestic blender for 2 minutes. For proteolytic hydrolysis, the samples were kept at 50 ^{o}C with addition of water at ratio 1/2 (w/v) and Alcalse at ratio 0.5 % (w/w). After hydrolysis, the samples was filtered to collect the filtrate and residues. The filtrate was centrifuged. The precipates containing carotenoids was separated and extracted with mixture of 3 hexane and 1 acetone at ratio 4/1 with 2 repeated cycles. The residues after filtration was dried and used for extraction according to procedure as for dried shrimp waste without enzymatic pretreatment.

2.2.2. Solvent extraction of carotenoids

The total carotenoids (as astaxanthin) were extracted using a mixture of acetone and hexane with the ratio of solvent: shrimp waste 3/1 (v/w). The ratio of hexane: acetone in the mixture

was varied from 1 : 2, 1 : 1, 1 : 2, 1 : 3 and 1 : 4. The temperature of extraction mixture were kept at 40, 50, 60 and 70 ^{o}C for two hours with or without vortexing. For extraction, 2 grams of shrimp waste were used. The extraction was caried out with 2 repeated cycles. Each experiment was done in triplicate.

2.2.3. Quantification of carotenoids

After extraction, the mixture was centrifuged to collect the supernatant. Then 1 ml supernatant was dried by evaporation to remove the solvent and the residues were resuspended in 2.5 ml mixture of hexane and acetone at ratio 3:1. The amount of the carotenoids, reported as astaxanthin, was quantified by absorbance measurement at 470 nm using Astaxanthin at concentration from 1 μg/mL to 8 μg/mL (Sigma) as standard [1].

2.2.4. Identification of astaxanthin by thin layer chromatography (TLC)

The extracted carotenoids in supernatant were visualised by TLC according to Dalei and Sahoo with small modification. For this, a small volume of the extract was spotted on silica gel plate and developed using acetone: hexane 1:3 (v/v) [9]. The separated bands were identified using standard astaxanthin (Sigma).

3. RESULTS AND DISCUSSION

3.1. Optimization conditions for carotenoid extraction

According to Sachindra, the mixture of polar and non-polar solvent is beneficial since the polar solvents remove the water in tissues which will aid in the extractability of pigments in nonpolar solvents in subsequent extractions [1]. However the lower carotenoid yield in the mixture of hexane: acetone at ratio 1/1 was observed than in the mixture of isopropanol: hexane at the same ratio (38.5 μg/g compared to 43.9 μg/g). In this study, the ratio of hexane to acetone (v/v) were varied as of 1/1, 1/2, 1/3 and 1/4. The ratio of solvent to sample was kept 3/1 (v/w). The samples were pretreated by drying and milling. The extraction was carried out at 60 °C for 2 hours with vortexing every 15 min with three repeated cycles. The extracted carotenoids were quantified (Table 1).

Table 1. Effect of ratio hexane/acetone on the carotenoid yield.

Ratio hexane/acetone	**Carotenoid yield (μg astaxanthin/g waste d.b)**
1: 2	33.64 ± 4.76
1: 1	34.36 ± 6.96
2: 1	37.53 ± 0.87
3: 1	44.90 ± 0.16
4: 1	44.10 ± 17.20

The carotenoid yield increased when the ratio of hexane increased from 33 % to 75 % (corresponding to ratio hexane: acetone from 1/2 to 3/1). If ratio hexane to acetone increased further to 80 %, the slight decrease of carotenoid yield was observed. According to Sachindra, the hexane ratio for optimal extraction yield of carotenoids from fresh shrimp waste was 60%

[1]. The higher ratio of hexane 75 % in this study could be explained by lower water content of dry waste requires lower ratio of polar solvent acetone. Thus the ratio 3/1 was chosen for the next experiment. Compared to a reported study [1], the carotenoid yield of 44.9 µg/g from dried shrimp waste was reasonable.

In the next experiment the effect of extraction temperature, duration of extraction was investigated. Elevation of extraction temperature from 40 °C to 60 °C resulted in increase of carotenoid yield from 38.2 µg/g to 44.3 µg/g. Almost no increase of yield was observed at higher extraction temperature of 70 °C (Figure 1 A). Therefore, extraction temperature of 60 °C was chosen. The extraction yield showed optimal at extraction temperature of 70 °C when extracting shrimp waste by sunflower oil [2]. The extraction yield was increased from 35.4 µg/g to 44.5 µg/g when extraction time increased from 1 hour to 2 hours. Prolonging extraction time to 3 hours resulted in insignificant increase in yield (Figure 1 B). Thus two hours of extraction was optimal. Optimal carotenoid yield was found at extraction time of 150 min by Sachindra when using sunflower oil for extraction [2].

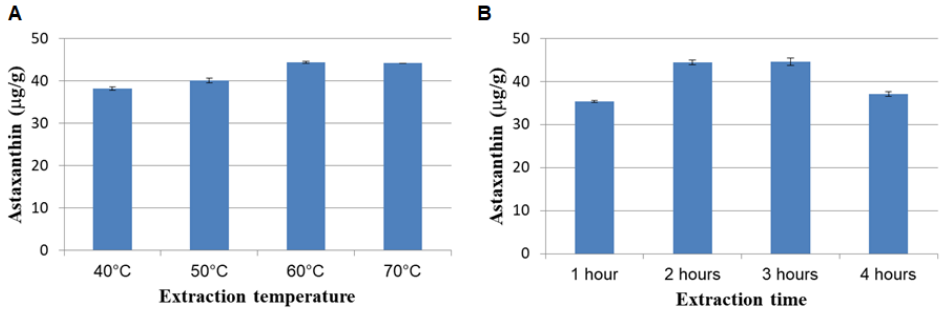

Figure 1. The effect of extraction temperature (A), extraction time (B) on extracted carotenoids yield.

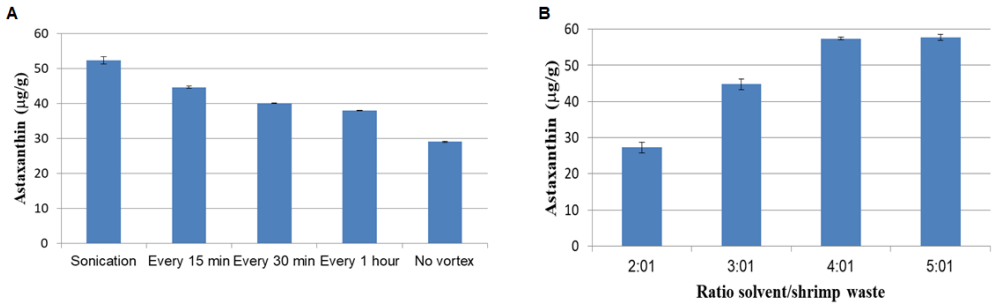

Figure 2. The effect of mixing (A) and ratio solvent/shrimp waste (B) on extracted carotenoids yield.

Mixing had strong effect on the carotenoid yield, where higher yield was achieved at the better mixing situation, indicating by frequency of vortexing. Mixing accelerates the diffusion and thus elevated mass transfer coefficient, leading to higher extraction yield. The yield could be 1.5 fold increased by vortexing every 15 min compared to no vortexing. However sonication gave the highest yield, reaching 52.4 µg/g, probably not only due to better mixing but might be due to enhancement of removing of carotenoids from shrimp shells (Figure 2A) [10].

The effect of ratio solvent to material was also studied (Figure 2B). The ratio of liquid to solid was varied from 2/1, 3/1, 4/1 to 5/1 (v/w). The higher ratio of solvent to material, the higher extraction yield. The yield was almost twofold increase when ratio of solvent doubled

from 2/1 to 4/1. However, the increase of ratio from 4/1 to 5/1 resulted in almost no increase of the yield. Therefore, the ratio of solvent to dried shrimp waste of 4/1 was optimal, giving carotenoid yield 57.4 μg/g. Sachindra found the optimal ratio of solvent to wet shrimp waste 5/1, a bit higher than dried shrimp waste [1]. Therefore, using dried shrimp waste could reduce a large volume of solvent due to reduction of water content and due to lower ratio of solvent to material.

3.2. Effect of pretreatment method

Firstly, the effect of cooking on extracted carotenoid yields was studied. After cooking, the shrimp waste was dried and grinded according to protocol. The results from Figure 3 showed that the sample with cooking gave lower yield (1.2- fold decrease from 45.4 μg/g to 37.3 μg/g). This was similar to the results reported by Mezzomo, who found the cooking resulted in decrease of extraction yield almost 1.24 times [8]. This could be explained by the releasing of carotenoids in the water, probably due to activating enzyme in shrimp waste. The red color of the cooked water confirmed this hypothesis. However, according to Mezzomo, although the extraction yield by cooking decreased 1.24 times, the total carotenoid content per gram extract increased 1.26 times. The authors suggested that the cooking process can break the carotenoid–protein complex, releasing the carotenoid compounds and facilitating its extraction.

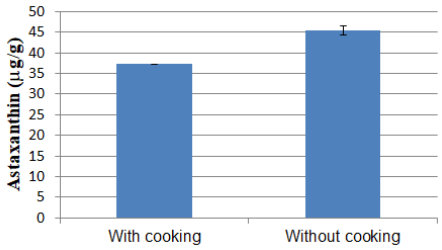

Figure 3. The effect of cooking on extracted carotenoids yield.

In the next experiment the effect of proteolytic hydrolysis time on extracted carotenoid yield was studied. Alcalase was used for this experiment. It could be seen that prolonging hydrolysis time resulted in higher carotenoid yield (Table 2).

The carotenoids in shrimp waste exit in the form of carotenoid–protein complex. Therefore, protease will hydrolyze this complex and release the carotenoids, facilitate extraction, leading to increase of carotenoid yield. The increase of carotenoid yield by proteolytic hydrolysis was reported also in [5]. Prolonging hydrolysis time to five hours, however, decreased the yield, probably due to degradation (Table 2). The increase of carotenoid yield was also observed when hydrolysis time prolonged from 3 to 5 hours by Chakrabarti [11]. Compared to the dried shrimp waste sample without hydrolysis, the carotenoid yield from fresh shrimp with hydrolysis was 2.6 fold increase (148.8 μg/g compared to 57.4 μg/g) (Table 2). It could be seen from Table 2, the carotenoid content almost removed from shrimp shells into the filtrate after only one hour of hydrolysis, remained at very low level in the residues. However, the hydrolysis of carotenoid-protein complex in filtrate continued, releasing carotenoid maximum at 4 hours of hydrolysis. The results of Table 2 also suggested that the extraction of carotenoids from residues could be skipped out, since their carotenoid contents accounted for only less than 6.6 % of total extracted carotenoids.

Table 2. Effect of time of enzymatic hydrolysis on the carotenoid yield.

Hydrolysis time (hours)	Carotenoid yield (μg astaxanthin/g waste d.b)		
	In filtrate	In residues	Total
1	94.12	10.40	104.52
2	121.88	10.96	132.85
3	132.47	9.94	142.41
4	139.06	9.73	148.80
5	106.07	10.19	116.26

The presence of astaxanthin in extract was confirmed by TLC (Figure 4). The results suggested that free astaxanthin was the major pigment in extracted carotenoids from dried shrimp waste of *Penaeus vannamei*. In contrast in marine shrimp *Penaus semisulcatus*, astaxanthin esters were the major carotenoids [12]. The components of carotenoids in extracts was reported to depend on type of shrimp, extraction method and TLC method [9, 12].

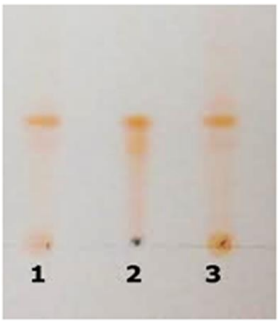

Figure 4. Chromatogram of extracted carotenoids from dried shrimp waste at ratio hexane: acetone 2:1 (1) and 3:1 (3), (2) Astaxanthin standard (Sigma).

4. CONCLUSION

The optimal conditions for astaxantin extraction from dried shrimp waste were using mixture of hexane and acetone at ratio 3/1, extraction temperature of 60 °C, extraction time of 2 hours, and ratio of solvent to dried shrimp waste 4/1. Ultrasound and vortexing positive resulted on extracted carotenoid yield. The extracted carotenoid yield decreased 1.2-fold by cooking pretreatment. Meanwhile the enzymatic hydrolysis could increase the extracted carotenoid yield up to 2.6-fold, reaching 148.8 μg/g raw material.

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